TRADE SECRET

Study Title

H-28072: Static, 72-Hour Growth Inhibition Limit Test with the Green Alga, *Pseudokirchneriella subcapitata*

TEST GUIDELINES: OECD Guideline for the Testing of Chemicals
Section 2 (Part 201) (2006)

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GOOD LABORATORY PRACTICE COMPLIANCE STATEMENT

This study was conducted in compliance with U.S. EPA TSCA (40 CFR part 792) Good Laboratory Practice Standards, which are compatible with current OECD and MAFF (Japan) Good Laboratory Practices.

Study Director: Terry Lee Sloman — 11 JUL 2008

Terry Lee Sloman, B.S.
Staff Ecotoxicologist

Date

QUALITY ASSURANCE STATEMENT

Work Request Number: 17199
Service Code Number: 280

Key inspections for DuPont work request 17199, service code 280 were performed for the tasks completed at DuPont by the Quality Assurance Unit of DuPont and the findings were submitted on the following dates.

Phase Audited	***Audit Dates***	***Date Reported to Study Director***	***Date Reported to Management***
Protocol:	June 1, 2007	June 1, 2007	June 1, 2007
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Report Revision 1:	July 8, 2008	July 8, 2008	July 8, 2008

Reported by: Donna M. Johnston — 11 July 2008
Donna M. Johnston
Quality Assurance Auditor
Date

CERTIFICATION

We, the undersigned, declare that this report provides an accurate evaluation of data obtained from this study.

Reviewed by: Robert A. Hoke — 9 July 2008

Robert A. Hoke, Ph.D.
Principal Research Ecotoxicologist and Manager

Date

Issued by Study Director: Terry Lee Sloman — 11 JUL 2008

Terry Lee Sloman, B.S.
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Date

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STUDY INFORMATION

Substance Tested:
- HFPO Dimer Acid Ammonium Salt
- 2,3,3,3-tetrafluoro-2-(heptafluoropropoxy)propionic acid, ammonium salt
- 62037-80-3 (CAS Number)
- H-28072

Haskell Number: 28072

Composition:

82.6%	Ammonium 2,3,3,3-tetrafluoro-2-(heptafluoropropoxy)propionate*
13.9%	Water
3.5%	Ammonium
0.41%	Organic Impurities

* Note: The Ammonium-2,3,3,3-tetrafuoro-2-(heptafluoropropoxy) propionate component (HFPO Dimer ammonium salt) contains 0.1 ppm HFPO trimer ammonium salt.

Purity: See composition, above

Physical Characteristics: Clear and colorless concentrated aqueous solution

Stability: The test substance appeared to be stable under the conditions of the study; no evidence of instability was observed.

Study Initiated/Completed: May 25, 2007 / (see report cover page)

Experimental Start/Termination: June 5, 2007 / June 8, 2007

REASON FOR REVISION 1

To provide consistent reporting of endpoints among studies based on guidance contained in OECD TG 201, 202 and 203.

SUMMARY

The toxicity of H-28072 to the green algae, *Pseudokirchneriella subcapitata*, was determined in a 72-hour, static limit test. The test was conducted in accordance with OECD Guideline for the Testing of Chemicals: 201 (2006).

The purity of H-28072 was 82.6% by analysis. The study was conducted with a blank control and a nominal limit test concentration of 120 mg/L H-28072 (106 mg/L mean, measured) at a mean lighting intensity of 5890 lux (range of 5650 to 6080 lux), a mean temperature of 23.9°C (range of 23.8 to 24.0°C), and a shaking speed of 95 rpm. The mean, measured limit test concentration was 80-120% of the nominal limit test concentration for the study. Synthetic algal-assay-procedure (AAP) nutrient medium was used as the test diluent and blank control. Test solutions were not renewed. Six replicates were used for the limit test concentration and the blank control. A single test flask was used for the abiotic (stability) control. Healthy cell count, area under the growth curve, and growth rate were determined at 24-hour intervals over the 72-hour test.

Inhibition of cell growth expressed as biomass (cell number), area under the growth curve, and average specific growth rate of *Pseudokirchneriella subcapitata* exposed to a nominal limit test concentration of 120 mg/L H-28072 for 72 hours was -2, -4, and 0%, respectively.[a] Healthy cell counts increased in the blank control by at least a factor of 16 in 72 hours, the coefficient of variation of average specific growth rates during the whole test period (0-72 hours) in blank control replicates did not exceed 7%, and the mean coefficient of variation for section-by-section specific growth rates (days 0-1, 1-2, and 2-3) in the blank control replicates did not exceed 35%, thereby satisfying the appropriate test acceptance criteria. The nominal H-28072 concentrations in the limit test concentration and abiotic control were each 120 mg/L. The Day 0 measured concentrations were 105 and 105 mg/L, and the 72-hour measured concentrations were 107 and 109 mg/L, respectively.

No significant inhibition was seen at the nominal limit test concentration of 120 mg/L H-28072. The E_bC_{50} (0-72 hour) values, based on the nominal limit test concentration and cell count and area under the growth curve were both greater than 120 mg/L. The E_rC_{50} (0-72 hour) value, based on the nominal limit test concentration and growth rate was greater than 120 mg/L. The LOEC values, based on the nominal limit test concentration and cell count, area under the growth curve, and growth rate, were each greater than 120 mg/L H-28072. The NOEC values,

[a] Negative values indicate stimulation of growth.

based on the nominal limit test concentration and cell count, area under the growth curve, and growth rate, were each 120 mg/L H-28072.[a]

The results are summarized as follows:

Nominal concentrations of H-28072, mg/L	Blank control, 120 (limit test concentration), and abiotic control (120)
Day 0 measured concentrations of H-28072, mg/L	ND,* 105, and 105
72-hour measured concentrations of H-28072, mg/L	ND,* 107, and 109
Mean, measured concentrations of H-28072, mg/L	ND,* 106 (limit test concentration), and 107 (abiotic control)
E_bC_{50} (0-72 hour) for H-28072, based on nominal concentration, mg/L	Cell Count: greater than 120
	Area Under Curve: greater than 120
E_rC_{50} (0-72 hour) for H-28072, based on nominal concentration, mg/L	Growth Rate: greater than 120
72-hour LOEC for H-28072, based on nominal concentration, mg/L	Cell Count: greater than 120
	Area Under Curve: greater than 120
	Growth Rate: greater than 120
72-hour NOEC for H-28072, based on nominal concentration, mg/L	Cell Count: 120
	Area Under Curve: 120
	Growth Rate: 120

* ND denotes not detected. The limit of detection for H-28072 was calculated as 0.0001 µg/L for day 0 and day 3.

[a] The E_bC_{50} (0-72 hour) is defined as the "effective concentration" producing a 50% inhibition of growth based on the 72-hour cell count (density) or area under the growth curve relative to the control. The E_rC_{50} (0-72 hour) is defined as the "effective concentration" producing a 50% inhibition of growth based on the 0-72 hour growth rate relative to the control. The LOEC is defined as the lowest concentration of test substance that had a significant effect on the measured parameter relative to the control. The NOEC is defined as the highest concentration of test substance that had no significant effect on the measured parameter relative to the control.

INTRODUCTION

The objective of this study was to assess the toxicity of H-28072 to the green algae, *Pseudokirchneriella subcapitata,* during a static, 72-hour limit test.

MATERIALS AND METHODS

A. Test Guidelines

The study design complied with the following test guidelines:

- OECD, Section 2 (Part 201): Effects on Biotic Systems, *Guideline for the Testing of Chemicals* (2006).

B. Test Substance

The test substance, H-28072, was supplied by the sponsor. The test substance contained 82.6% H-28072 active ingredient by analysis (Appendix A). Test substance solubility and stability were confirmed under study conditions based on analytical measurements.

C. Test Solution Preparation

A primary stock solution with a nominal concentration of 120 mg/L H-28072 was prepared by dissolving approximately 120 mg of H-28072 in 1000 mL of filter-sterilized AAP nutrient medium. The nominal 120 mg/L primary stock solution was used as the limit test concentration and as the abiotic (stability) control solution. Filter-sterilized AAP nutrient medium was used as the blank control solution. The blank control and limit test concentration solutions were clear and colorless with no visible precipitate.

D. AAP Nutrient Medium Preparation

To prepare one liter of AAP nutrient medium, 1 mL of each of the 6 macronutrient stock solutions and 1 mL of the micronutrient stock solution are added to approximately 800 mL of Milli-Q® (deionized) water with mixing after each addition.(1) The final volume of the medium is brought to 1 liter with additional Milli-Q® water. The larger volume required for the definitive test was prepared based on these proportions.

The nutrient medium pH was adjusted to 7.54 with 0.1N hydrochloric acid and filter-sterilized through 0.22 µm cellulose acetate filters into sterile containers. The containers with the resulting filter-sterilized AAP nutrient medium were stored in the refrigerator in the dark at approximately 4°C and acclimated to ambient temperature prior to use.

E. Organism Culture

Pseudokirchneriella subcapitata, a freshwater, unicellular, non-motile, green alga, was used in this study.

Pseudokirchneriella subcapitata was cultured and maintained at Haskell Laboratory. The original culture source was the Department of Botany - Culture Collection of Algae - The University of Texas at Austin, Austin, Texas 78713-7640.

The culture method for *P. subcapitata* was based on published literature.[1,2] Prior to the study, *P. subcapitata* cultures were maintained under photoperiod, shaking speed, and temperature conditions similar to those used in the study. Illumination was maintained at 5005 ± 805 lux. The organisms were cultured in sterilized 250-mL Erlenmeyer flasks containing approximately 50 mL of filter-sterilized AAP nutrient medium and were aseptically transferred to fresh medium every 3 to 7 days. The flasks were fitted with sterilized foam stoppers to permit gas exchange. The *P. subcapitata* culture used to inoculate test vessels was aseptically transferred to fresh medium 4 days prior to use.

F. Study Methods

The study design used for the limit test is described below.

Organism	Nutrient Medium	Flask Volume (mL)	Solution Volume (mL)	Volume Ratio
Pseudokirchneriella subcapitata	AAP	250	50	5:1

The **target** environmental parameters are described below.

Initial Population	Illumination (lumens/m² = lux)	Photoperiod (hours)	Shaking Speed (rpm)	Temperature (°C)
10,000 cells/mL	4440 to 8880	24	100	21 to 24

Test chambers were sterilized 250-mL Erlenmeyer flasks containing approximately 50 mL of test solution. Flasks were fitted with sterilized foam stoppers to permit gas exchange. Test flasks were randomly positioned on a shaker table with a shaking speed of approximately 100 rpm in an environmental chamber at 21 to 24°C controlled at ± 2°C and were repositioned daily. Air temperature in the environmental chamber was measured daily and recorded with a continuous temperature chart recorder. Flasks were illuminated continuously using cool-white fluorescent tubes.

Pseudokirchneriella subcapitata growth was determined by counting the number of cells in an approximate 0.2-mL sample from each flask at approximately 24, 48, and 72 hours after the definitive test initiation. The counts were conducted using a hemacytometer and a compound microscope. An aliquot of each sample was loaded into the 2 grid areas of the hemacytometer. All healthy cells located in 8 squares from each grid area (16 total squares) were counted and recorded in the study records. The total number of cells counted was multiplied by 10,000 to

determine the number of cells per milliliter. Counts were made at approximately the same time each day.

One limit test concentration, a culture medium blank control, and an abiotic control were used in this study. The nominal concentration of 120 mg/L H-28072 was chosen for the definitive test based on the results of a preliminary range-finding test. Test organisms were exposed for 72 hours without test medium renewal.

The blank control and the limit test concentration were tested as 6 replicates. The abiotic control was tested as a single flask (no replicates). Each flask, excluding the abiotic control, was randomly assigned a number to position the test flasks on the shaker table and to eliminate bias while counting. Flasks, excluding the abiotic control, were inoculated with approximately 10,000 *P. subcapitata* cells/mL by aseptically transferring 0.234 mL of algal inoculum from a pre-counted, logarithmically growing stock culture to each flask.

Measurements of pH were conducted at test start in an aliquot taken directly from the appropriate mixing vessel and at 72 hours in an aliquot taken after pooling all replicates, where appropriate, of each control or test concentration.

G. Sample Preparation and Chemical Analysis

1. Sample Collection

A full description of sample preparation and chemical analysis is presented in Appendix B.

Samples and back-up samples from test solutions containing H-28072 at a nominal concentration of 120 mg/L and the blank control were transported on ice from Haskell Laboratory to the analytical laboratory for concentration verification on each test day (day 0 and day 3).

Concentrations of H-28072 were measured by high performance liquid chromatography with detection by mass spectrometry (LC/MS/MS).

H. Statistical Analyses

The data were analyzed to determine the toxicity of H-28072 in terms of its no-observed-effect concentration (NOEC), lowest-observed-effect concentration (LOEC), and concentration causing a 50% inhibition of growth relative to the control (EC_{50}). The NOEC is defined as the highest concentration of test substance that had no significant effect on the measured parameter relative to the blank control. The LOEC is defined as the lowest concentration of test substance that had a significant effect on the measured parameter. The E_bC_{50} (0-72 hour) is defined as the effect concentration producing a 50% inhibition of growth based on the 72-hour healthy cell count or area under the growth curve. The E_rC_{50} (0-72 hour) is defined as the effect concentration producing a 50% inhibition of growth based on the 72 hour growth rate based on healthy cell count. Endpoints evaluated were healthy cell count, area under the growth curve, and growth rate based on healthy cell count. Analyses are reported based on the nominal H-28072 concentration and were conducted using SAS version 8.2.[(3)]

The area under the growth curve for each interval was calculated according to the following formula:

$$A = [0.5 \times t_1 \times (N_1 - N_0)] + [0.5 \times (t_2 - t_1) \times (N_2 + N_1 - 2N_0)] + \ldots + [0.5 \times (t_n - t_{n-1}) \times (N_n + N_{n-1} - 2N_0)]$$

where t_1, t_2, t_{n-1}, and t_n are times of observation measured from the initiation of the study and N_0, N_1, N_{n-1}, and N_n are the initial and subsequent healthy cell counts (cells/mL) corresponding to the observation times.

The average specific growth rate (μ) for a specific period was calculated as the logarithmic increase in the biomass for each test concentration and control according to the following formula:

$$\mu_{i-j} = \frac{\ln X_j - \ln X_i}{t_j - t_i} \ (\text{day}^{-1})$$

where μ_{i-j} is the average specific growth rate from time (t) i to j; X_i is the healthy cell count (cells/mL) at time i; and X_j is the healthy cell count at time j.

The means and standard errors of the healthy cell counts, area under the growth curves, and growth rates for each test concentration and the blank control were calculated using standard procedures.[4] The coefficient of variation (CV) is a dimensionless measure of the variability of a parameter, calculated as the ratio of the standard deviation (SD) to the mean (X), and expressed as a percent value according to the following formula:

$$\% \ CV = \frac{SD}{X} \times 100$$

The percent growth inhibition, % I, was calculated according to the following formula:

$$\% \ I = \frac{C - T}{C} \times 100$$

where C is equal to the mean of a given measurement variable (i.e., healthy cell count, area under the growth curve, or growth rate based on healthy cell count) for the blank control at a selected sampling interval and T is the mean of the measurement variable for a test concentration at the selected sampling interval. Negative values of inhibition indicate stimulation of growth.

Continuous data were evaluated for normality using the Shapiro-Wilk test[5] while homogeneity of variance was assessed by Levene's test.[6] Data found to be both normal and homogenous were evaluated using ANOVA.[7] Data consistent with a monotonic concentration-response were evaluated using the Jonckheere -Terpstra trend test.[8] Data not consistent with a monotonic concentration-response were assessed using the Dunnett method[9] if the data were normally distributed and homogeneous, and using the Tamhane-Dunnett (or T3) method[7] if the data were normally distributed but heterogeneous. Non-normal data were analyzed by alternative methods.[10] If possible, a transformation was found to obtain normality. Otherwise, a Kruskal-

Wallis test[8] replaced the ANOVA, Dunn's test[7] replaced Dunnett's, and the Jonckheere-Terpstra test was still applicable, except as noted. Outliers were determined by the Tukey outlier rule[11] and, if present, their effect on the conclusions was determined. All statistical tests were calculated at a significance level of $p = 0.05$.

If massive ties were present in a continuous response, exact permutation data analysis methods[11] were used for analysis. An exact version of the Kruskal-Wallis test was used. Exact versions of Jonckheere-Terpstra or separate Mann-Whitney[8] (or, equivalently, Wilcoxon) tests comparised each treatment group to the blank control, using a Bonferonni correction.[7]

The percent inhibition at the single limit test concentration and the corresponding 95% confidence interval were calculated using a single-step test procedure. The null hypothesis tested, using the MAXSD test[12], was that growth inhibition was greater than or equal to 50%.

RESULTS AND DISCUSSION

A. Analytical Report

A full description of the results, including representative chromatograms, is presented in Appendix B. The measured concentrations of H-28072 in the day 0 limit test concentration and abiotic control solutions were 106% of the targeted nominal test concentrations adjusted for test substance purity of 82.6%. The measured concentrations of H-28072 in the day 3 test solutions were 108 and 110%, respectively, of the targeted nominal test concentrations adjusted for test substance purity of 82.6%. The mean, measured concentrations of H-28072 in the test solutions were 107 and 108% respectively, of the targeted nominal test concentrations adjusted for test substance purity of 82.6%. The blank control solutions contained no detectable concentrations of H-28072 on either day 0 or day 3.

B. In-Life Report

The nominal limit test concentration for the definitive test was 120 mg/L H-28072. The nominal concentration of the abiotic control was 120 mg/L H-28072. A culture medium blank control was used in this study. The nominal H-28072 concentrations (based on 82.6% H-28072 active ingredient) in the 120 mg/L and abiotic control solutions were each 99.1 mg/L. The corresponding mean, measured concentrations were 106 and 107 mg/L. The mean, measured concentrations were within 80-120% of the nominal limit test concentration for the study.

All environmental parameters for the definitive test (Tables 1 and 2) were within expected ranges. During the test, the shaking speed was 95 rpm, pH ranged from 7.01 to 7.83, mean lighting was 5890 lux with a range of 5650 to 6080 lux, and temperature in the environmental chamber ranged from 23.8 to 24.0°C.

Data on healthy cell count, area under the growth curve, and growth rate are presented in Tables 3, 4, and 5, respectively. Growth curves for the blank control solution and test solution are presented in Figure 1. Healthy cell counts increased in the blank control by a factor of approximately 240 in 72 hours, the mean coefficient of variation for section-by-section specific growth rates in the blank control was 17.71%, and the coefficient of variation of the average specific growth rate during the 72-hour exposure period in the blank control replicates was 1.87%, thereby satisfying the appropriate test acceptance criteria.

Inhibition of growth based on healthy cell count, area under the growth curve, and growth rate of *P. subcapitata* exposed to a nominal limit test concentration of 120 mg/L H-28072 for 72 hours was -2, -4, and 0%, respectively. A summary of the 72-hour EC_{50}, LOEC, and NOEC values is presented in Table 6. The E_bC_{50} (0-72 hour) and E_rC_{50} (0-72 hour) values, based on the nominal concentration and cell count, area under the growth curve, and growth rate, were each greater than 120 mg/L. The 72-hour LOEC values, based on the nominal concentration and cell count, area under the growth curve, and growth rate, were each greater than 120 mg/L. The 72-hour NOEC values, based on the nominal concentration and healthy cell count, area under the growth curve, or growth rate, were each 120 mg/L.

C. Statistical Report

The data for healthy cell count, and growth rate based on healthy cell count were determined to be normally distributed (Shapiro-Wilk test[5]) with equal variances (Levene's test[6]). Therefore, the Jonckheere-Terpstra[8] trend test was used to determine the LOEC and NOEC values. The data for area under the growth curve were determined to be non-normally distributed (Shapiro-Wilk test[5]). Therefore, a non-parametric analysis was performed (Kruskal-Wallis test[8]) and the Jonckheere-Terpstra test[8] was used to determine the LOEC and NOEC values. No outliers were found[11] in the data for healthy cell count, area under the growth curve, and growth rate based on healthy cell count. The LOEC and NOEC values for healthy cell count, area under the growth curve, and growth rate based on healthy cell count were determined to be >120 mg/L and 120 mg/L, respectively.

The MAXSD test[12] was used to test the null hypothesis that growth inhibition was greater than or equal to 50%. The growth inhibition at the limit test concentration was also not found to be statistically significantly different from the blank control, and therefore the E_bC_{50}, and E_rC_{50} values for healthy cell count, area under the growth curve, and growth rate based on healthy cell count were determined to be greater than 120 mg/L.[3]

CONCLUSIONS

H-28072 was assessed for toxicity to *Pseudokirchneriella subcapitata* in a static 72-hour test. There was no significant inhibitory effect on the growth and reproduction of *Pseudokirchneriella subcapitata* when exposed to a nominal concentration of 120 mg/L H-28072 for 72 hours. Therefore, the E_bC_{50} (0-72 hour) and E_rC_{50} (0-72 hour) values, based on the nominal concentration and cell count, area under the growth curve, or growth rate, are greater than 120 mg/L H-28072. The 72-hour LOEC values, based on the nominal concentration and cell count, area under the growth curve, and growth rate, are each greater than 120 mg/L H-28072. The 72-hour NOEC values, based on the nominal concentration and healthy cell count, area under the growth curve, or growth rate, are each 120 mg/L.

RECORDS AND SAMPLE STORAGE

Specimens (if applicable), raw data, the protocol, amendments (if any), and the final report will be retained at Haskell Laboratory, Newark, Delaware, or at Iron Mountain Records Management, Wilmington, Delaware.

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TABLES

Table 1
pH Measurements of Test Solutions

Nominal H-28072 Concentration	pH	
	0-Hour (Day 0)	72-Hour (Day 3)
Blank Control	7.61	7.83
120 mg/L	7.30	7.01
Abiotic Control: 120 mg/L	7.30	7.25

Table 2
Test Conditions: Chamber Light Intensity, Shaking Speed, and Temperature Range

Mean Light Intensity at Test Initiation[a] (lux)	Light Intensity Range at Test Initiation (lux)	Oscillations (rpm)	Temperature (°C)
5890	5650 to 6080	95	23.8 to 24.0

a mean of 3 measurements

Table 3
Healthy Cell Count Data Summary

Nominal H-28072 Concentration mg/L	Rep.	Exposure Initiated: Day 0: 05 June 2007		Exposure Ended: Day 3: 08 June 2007	
		Healthy Cells/mL Count by Test Day			
		0	1	2	3
Blank Control	1	10,000	90,000	400,000	2,170,000
	2	10,000	60,000	410,000	2,610,000
	3	10,000	70,000	380,000	2,630,000
	4	10,000	40,000	490,000	2,390,000
	5	10,000	40,000	570,000	2,310,000
	6	10,000	60,000	340,000	2,030,000
Mean		10,000	60,000	431,667	2,356,667
Std. Dev.		0	18,974	83,766	238,216
Coeff. of Variation		0.0	31.6	19.4	10.1
120	1	10,000	80,000	300,000	2,270,000
	2	10,000	30,000	270,000	2,350,000
	3	10,000	40,000	650,000	2,240,000
	4	10,000	50,000	490,000	2,550,000
	5	10,000	50,000	570,000	2,520,000
	6	10,000	60,000	590,000	2,480,000
Mean		10,000	51,667	478,333	2,401,667
Std. Dev.		0	17,224	158,545	132,878
Coeff. of Variation		0.0	33.3	33.1	5.5
% Inhibition		0	14	-11	-2

Table 4
Area Under the Growth Curve Data Summary

Nominal H-28072 Concentration mg/L	Rep.	Exposure Initiated: Day 0: 05 June 2007 — Area Under the Growth Curve Based on Healthy Cells/mL Count by Test Day: Day 0-1	Day 0-2	Exposure Ended: Day 3: 08 June 2007 — Day 0-3
Blank Control	1	40,000	275,000	1,550,000
	2	25,000	250,000	1,750,000
	3	30,000	245,000	1,740,000
	4	15,000	270,000	1,700,000
	5	15,000	310,000	1,740,000
	6	25,000	215,000	1,390,000
Mean		25,000	260,833	1,645,000
Std. Dev.		9,487	32,158	145,705
Coeff. of Variation		37.9	12.3	8.9
120	1	35,000	215,000	1,490,000
	2	10,000	150,000	1,450,000
	3	15,000	350,000	1,785,000
	4	20,000	280,000	1,790,000
	5	20,000	320,000	1,855,000
	6	25,000	340,000	1,865,000
Mean		20,833	275,833	1,705,833
Std. Dev.		8,612	78,893	185,995
Coeff. of Variation		41.3	28.6	10.9
% Inhibition		17	-6	-4

Table 5
Growth Rate Data Summary

Nominal H-28072 Concentration mg/L	Rep.	Exposure Initiated: Day 0: 05 June 2007 / Exposure Ended: Day 3: 08 June 2007 Growth Rate Based on Healthy Cells/mL Count by Test Day		
		Day 0-1	Day 0-2	Day 0-3
Blank Control	1	2.20	1.84	1.79
	2	1.79	1.86	1.85
	3	1.95	1.82	1.86
	4	1.39	1.95	1.83
	5	1.39	2.02	1.81
	6	1.79	1.76	1.77
Mean		1.75	1.88	1.82
Std. Dev.		0.32	0.09	0.03
Coeff. of Variation		18.19	4.97	1.87
120	1	2.08	1.70	1.81
	2	1.10	1.65	1.82
	3	1.39	2.09	1.80
	4	1.61	1.95	1.85
	5	1.61	2.02	1.84
	6	1.79	2.04	1.84
Mean		1.60	1.91	1.83
Std. Dev.		0.34	0.19	0.02
Coeff. of Variation		21.04	9.79	1.01
% Inhibition		9	-2	0

Table 5
Growth Rate Data Summary (continued)

Nominal H-28072 Concentration mg/L	Rep.	Exposure Initiated: Day 0: 05 June 2007 / Exposure Ended: Day 3: 08 June 2007 — Growth Rate Based on Healthy Cells/mL Count by Test Day					
		Day 0-1	Day 1-2	Day 2-3	Mean	Std. Dev.	Co of Var
Blank Control	1	2.20	1.49	1.69	1.79	0.36	20.28
	2	1.79	1.92	1.85	1.85	0.07	3.51
	3	1.95	1.69	1.93	1.86	0.14	7.73
	4	1.39	2.51	1.58	1.83	0.60	32.72
	5	1.39	2.66	1.40	1.81	0.73	40.23
	6	1.79	1.73	1.79	1.77	0.03	1.79
Mean		1.75	2.00	1.71	1.82		17.71
Std. Dev.		0.32	0.47	0.19	0.03		
Coeff. of Variation		18.19	23.63	11.38	1.87		
120	1	2.08	1.32	2.02	1.81	0.42	23.35
	2	1.10	2.20	2.16	1.82	0.62	34.33
	3	1.39	2.79	1.24	1.80	0.86	47.43
	4	1.61	2.28	1.65	1.85	0.38	20.44
	5	1.61	2.43	1.49	1.84	0.52	27.94
	6	1.79	2.29	1.44	1.84	0.43	23.22
Mean		1.60	2.22	1.67			29.45
Std. Dev.		0.34	0.49	0.36			
Coeff. of Variation		21.04	21.94	21.56			
% Inhibition		9	-11	2			

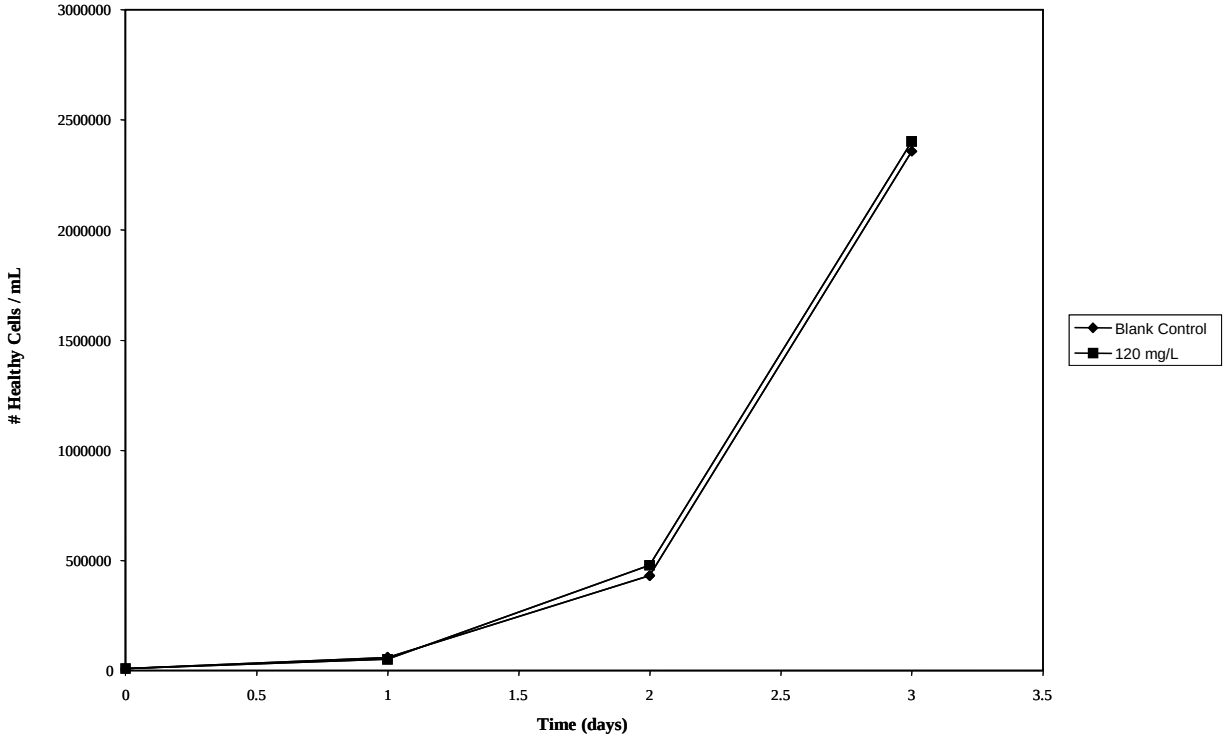
Table 6
72-Hour EC_{50}, LOEC, and NOEC Values for *Pseudokirchneriella subcapitata* Based on the Nominal Concentration of H-28072

Parameter	72-Hour E_xC_{50}*	Model	72-Hour LOEC	72-Hour NOEC	Method
Healthy Cell Count	> 120 mg/L	MAXSD	> 120 mg/L	120 mg/L	t-test
Area Under the Growth Curve	> 120 mg/L	MAXSD	> 120 mg/L	120 mg/L	t-test
Growth Rate	> 120 mg/L	MAXSD	> 120 mg/L	120 mg/L	t-test

* x = b for healthy cell count and area under growth curve; r for growth rate.

FIGURE

Figure 1
Healthy Cell Count Versus Time for *Pseudokirchneriella subcapitata* Based on the Nominal Concentration of H-28072



APPENDICES

Appendix A
Certificate of Analysis

E. I. du Pont de Nemours and Company
Wilmington. DE 19898
USA

CERTIFICATE OF ANALYSIS

This Certificate of Analysis fulfills the requirement for characterization of a test substance prior to a study subject to GLP regulations. It documents the identity and content of the test substance. This work was conducted under EPA Good Laboratory Practice Standards (40 CFR 792).

Haskell Code Number	H-28072
Common Name	HFPO Dimer Acid Ammonium Salt
Purity Percent	82.6%
Other Components	Water – 13.9% Ammonium (excess) – 3.5%
Date of Analysis	July 19, 2007
Recommended reanalysis interval	1 year
Instructions for storage	NRT&H
Reference	DuPont-23285
Analysis performed at	E. I. DuPont de Nemours and Company DuPont Haskell Laboratories Newark, Delaware USA

Peter A. Bloxham, Ph.D.
Analyst's Name

Analyst's signature

23-JUL-2007
Date

Revision #1
July 20, 2007

Appendix B
Analytical Report

Test Solutions Analyses:

H-28072: Static, 72-Hour Growth Inhibition Limit Test with the Green Alga,
Pseudokirchneriella subcapitata

AUTHOR: Karen M. L'Empereur, Ph.D.

ANALYTICAL STUDY COMPLETED ON: July 19, 2007
REPORT REVISED ON: August 17, 2007

PERFORMING LABORATORY: Critical Path Services (CPS)
3521 Silverside Rd.
Quillen Bldg., Suite 1-I
Wilmington DE 19810

SPONSOR: E.I. du Pont de Nemours and Company
Wilmington, DE 19898

CPS PROJECT NUMBER: 07-CPS-019

SPONSOR PROJECT NUMBER DuPont-22911, Rev. 1

WORK REQUEST NUMBER: 17199

SERVICE CODE NUMBER: 280

GOOD LABORATORY PRACTICE COMPLIANCE STATEMENT

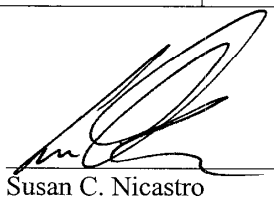
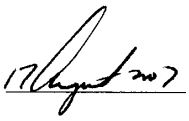
This analytical phase of this study was conducted in compliance with U.S. EPA TSCA (40 CFR Part 792) Good Laboratory Practice Standards, which are compatible with the OECD and MAFF Japan Good Laboratory Practice Standards.

CPS
Principal Investigator: Karen M. L'Empereur Date: 17-Aug-07
Karen M. L'Empereur, Ph.D.
Critical Path Services

QUALITY ASSURANCE STATEMENT

This study was inspected/audited by Quality Assurance according to CPS Standard Operating Procedures and EPA's Good Laboratory Practice Standards (40 CFR Part 792) and all findings were reported to the Study Director and Management. It was concluded that the final report accurately reflects the raw data for this study.

Phase Audited	Date of QAU Inspection	Date Reported to Study Director	Date Reported to Management
Sample Receipt	5-June-2007	5-June-2007	5-June-2007
Study Records, Final Report	2-July-2007	2-July-2007	2-July-2007
Revised Final Report	16-August-2007	17-August-2007	17-August-2007

CPS
Quality Assurance Auditor:  Date: 

Susan C. Nicastro
Critical Path Services

CERTIFICATION

We, the undersigned, declare that this report provides an accurate evaluation of data obtained from the analytical phase of this study.

CPS
Principal Investigator: _____ Date: 17-Aug-07
Karen M. L'Empereur, Ph.D.
Critical Path Services

CPS Management: _____ Date: 17 August '07
Julie E. Eble, Ph.D.
Critical Path Services
Laboratory Director

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SUMMARY

Samples from test solutions containing H-28072 at a nominal concentration of 120 mg/L and the blank control were submitted for concentration verification on test day 0. Samples from the pooled replicates of the blank control, test concentration, and an aliquot from the abiotic control were submitted at test end (day 3).

Concentrations of H-28072 in test solution samples were measured by high performance liquid chromatography with detection by mass spectrometry (LC/MS/MS).

The mean, measured concentration of H-28072 in the nominal 120 mg/L test concentration solution was 108% of the targeted nominal test concentrations adjusted for test substance purity of 82.6%.

The blank control solutions contained no detectable concentrations of H-28072 on either day 0 or day 3.

The report has been revised to reflect the change in the assigned purity of the H-28072 test substance from 81.4% to 82.6%. This change does not affect the conclusions of the study.

MATERIALS AND METHODS

A. Sample Preparation and Chemical Analysis

1. Sample Collection and Treatment

An aliquot from each of the blank control and test concentration were sampled at the study start (day 0) and submitted for analysis. An aliquot from the pooled replicates of the blank control, test concentration, and an aliquot from the abiotic control were sampled on day 3 and submitted for analysis. Back-up solutions also were provided for each sample. Samples and back-up samples were transported on ice to the analytical laboratory, and were stored refrigerated upon receipt and when not in use.

The samples, including controls, were diluted 2000x with a solution of HPLC grade water/acetonitrile, 50/50, v/v, before analysis. Dilution of the samples was necessary due the sensitivity of the detector to H-28072. Prior to dilution and analysis, aliquots from all day 3 samples were centrifuged for 15 minutes at a rate of 14,000 rpm to remove algae.

Concentrations of H-28072 were measured by high performance liquid chromatography with detection by mass spectrometry (LC/MS/MS) in samples that were stored refrigerated and analyzed within 1 day of sample receipt.

2. Instrument and Conditions

HPLC Instrument:	Agilent Model 1200
MS Instrument:	Applied Biosystems API 4000
Software:	Analyst 1.4.1

LC Parameters:

Column:	Phenomenex Luna C8; 150 x 2.0 mm, 5.0 µm
Mobile Phase:	Premix of water/acetonitrile, 50/50, v/v, with 0.15% acetic acid and 0.15% triethylamine
Flow Rate:	0.400 mL/min
Column Temperature:	30°C
Injection Volume:	3.0 µL

MS Parameters:

Polarity (+/-)	Q1 (m/z)	Q2 (m/z)	Dwell (msecs)	CUR (psi)	GS1 (psi)	GS2 (psi)	TEM (°C)	iHe	IS (V)	DP (V)	EP (V)	CE (V)	CXP (V)
-	329.00	285.00	100	40	11	60	400	on	-4500	-20	-10	-10	-5

3. Quantitation

A primary stock solution of the test compound, H-28072 (purity 82.6%), was made by dissolving the standard in water. Appropriate aliquots of the primary stock solution were diluted with AAP nutrient medium to prepare a secondary stock solution. On each day of analysis the secondary stock solution was diluted with a solution of water/acetonitrile, 50/50, v/v to prepare calibration standards at concentrations that bracketed the concentrations of the diluted test solutions. Duplicate injections of test and calibration standard solutions were made and peak areas were determined electronically.

The calibration standard curve was generated by regression analysis using the chromatographic peak areas of the calibration standard solutions. Data for test solutions were compared to the calibration standard curve to determine concentrations of H-28072. The limit of detection (LOD) was determined by calculating the average noise level in chromatograms of the blank control solutions and comparing them to the signal of a calibration standard of known concentration. Two chromatograms were examined for noise-related peaks near the retention time of the analyte. The LOD was calculated as 3 times the concentration equivalent of the mean noise level. The limit of quantitation (LOQ) was defined as the greater of 10 times concentration equivalent of the mean noise level or the lowest standard concentration.

RESULTS AND DISCUSSION

A. Analytical Report

1. Chromatographic Results

H-28072 eluted as a well-resolved chromatographic peak with a retention time of approximately 2.1 minutes. A typical calibration standard curve is shown in Figure 1. Representative chromatograms of a calibration standard solution, a blank control solution, and a test concentration solution are presented in Figures 2 to 4, respectively.

The LOD and LOQ were determined to be 0.0001 µg/L and 25.9 µg/L, respectively.

2. Test Solution Results

The measured concentration of H-28072 in the day 0 test concentration solution was 106% of the targeted nominal test concentration adjusted for test substance purity of 82.6% (Table 1). The measured concentrations of H-28072 in the day 3 test concentration and abiotic control solutions were 108 and 110%, respectively, of the targeted nominal test concentrations adjusted for test substance purity of 82.6% (Table 1). The blank control solutions contained no detectable concentrations of H-28072 on either day 0 or day 3.

The mean, measured concentrations of H-28072 in the nominal 120 mg/L test concentration and the abiotic control solutions were 106 and 107 mg/L, respectively.

H-28072 was determined to be stable over the course of the definitive test as evidenced by the analytical recoveries obtained from the day 0 and day 3 test concentration solutions.

TABLE 1

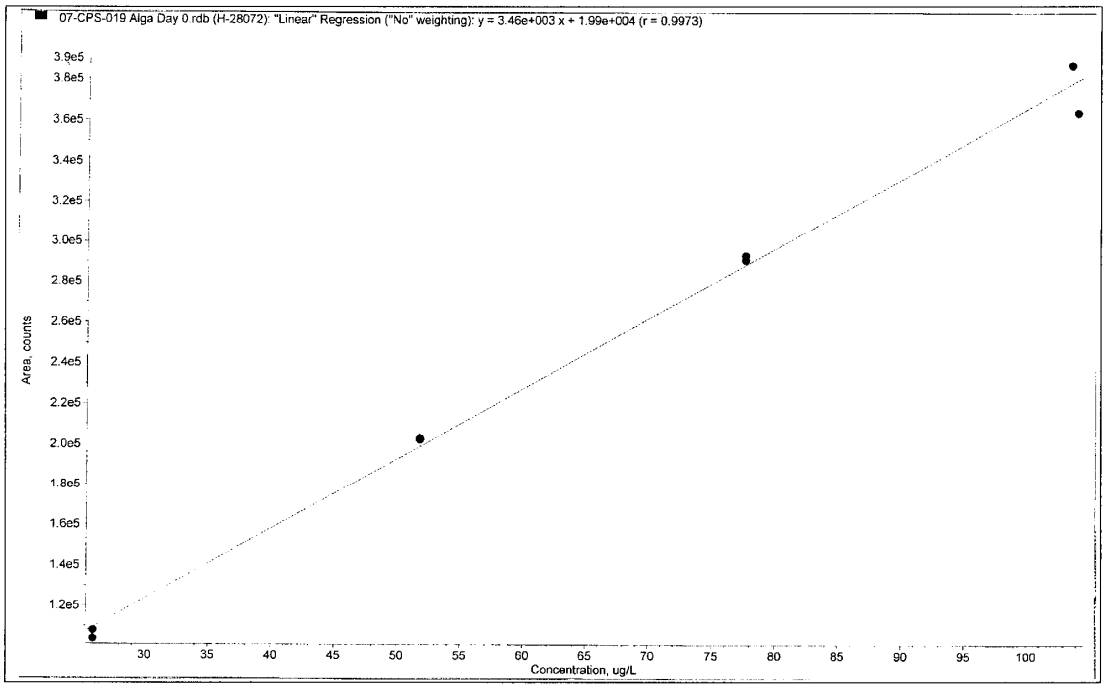
MEASURED CONCENTRATIONS OF H-28072 IN TEST SOLUTIONS

Nominal H-28072 Concentration (mg/L)[a]	Corrected H-28072 Concentration (mg/L)[b]	Measured H-28072 Concentration (mg/L) Day 0	Day 3	Mean, Measured H-28072 Concentration (mg/L)[c]	% Recovery[d]
Blank Control	0.0	ND[e]	ND	ND	---
120	99.1	105	107	106	107
Abiotic Control (120)	99.1	105[f]	109	107	108

a Defined in terms of H-28072/liter nutrient medium.
b Nominal H-28072 concentrations corrected for 82.6% purity by analysis.
c Calculated as (Day 0 measured concentration + Day 3 measured concentration)/2.
d Based on corrected H-28072 concentrations.
e ND denotes not detected. The limit of detection for H-28072 was calculated as 0.0001 µg/L.
f No separate analysis was conducted since the 120 mg/L test solution and the day 0 abiotic control solution were the same solution.

FIGURE 1

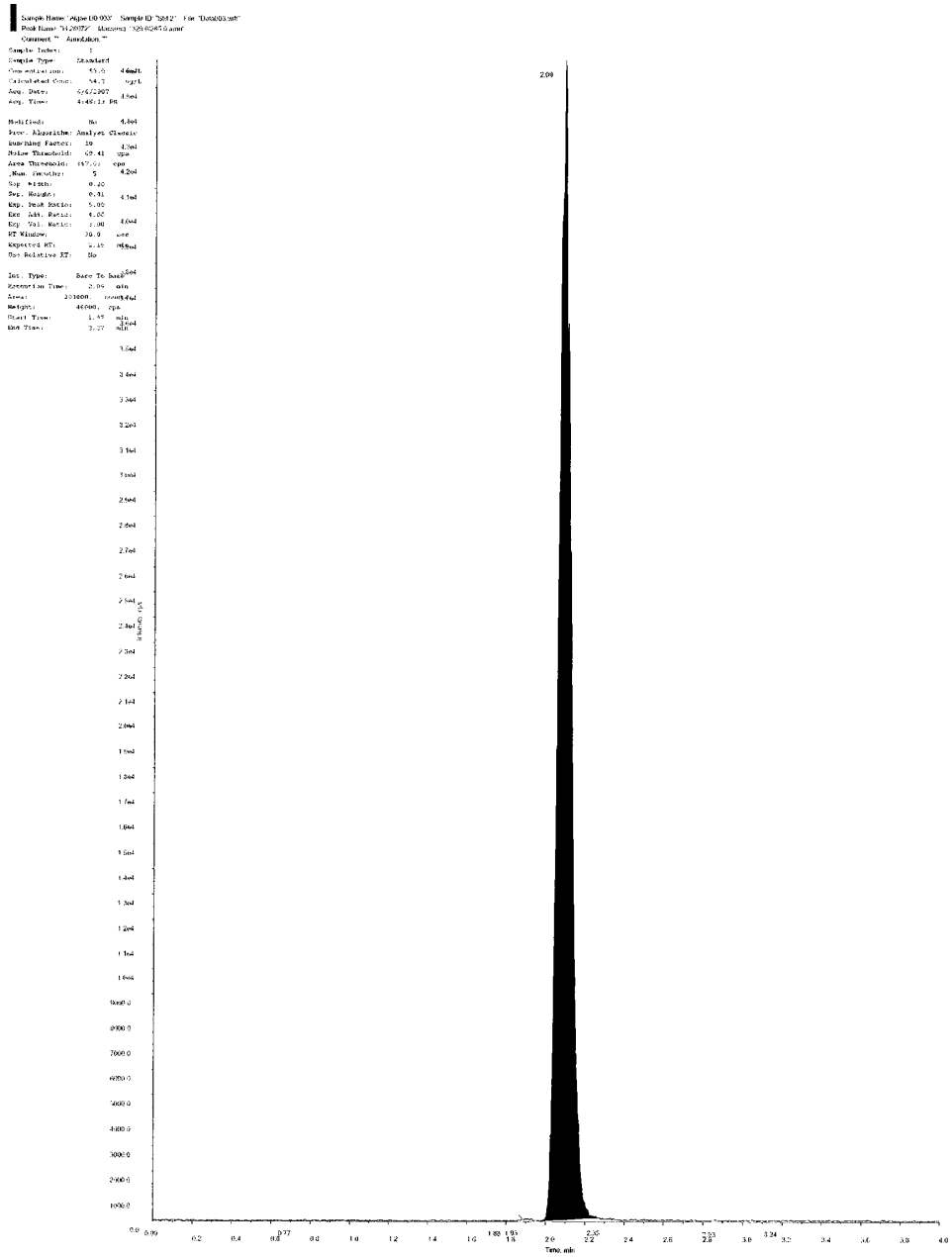
REPRESENTATIVE ANALYTICAL CALIBRATION STANDARD CURVE FOR H-28072



Concentration of H-28072 in µg/L

FIGURE 2

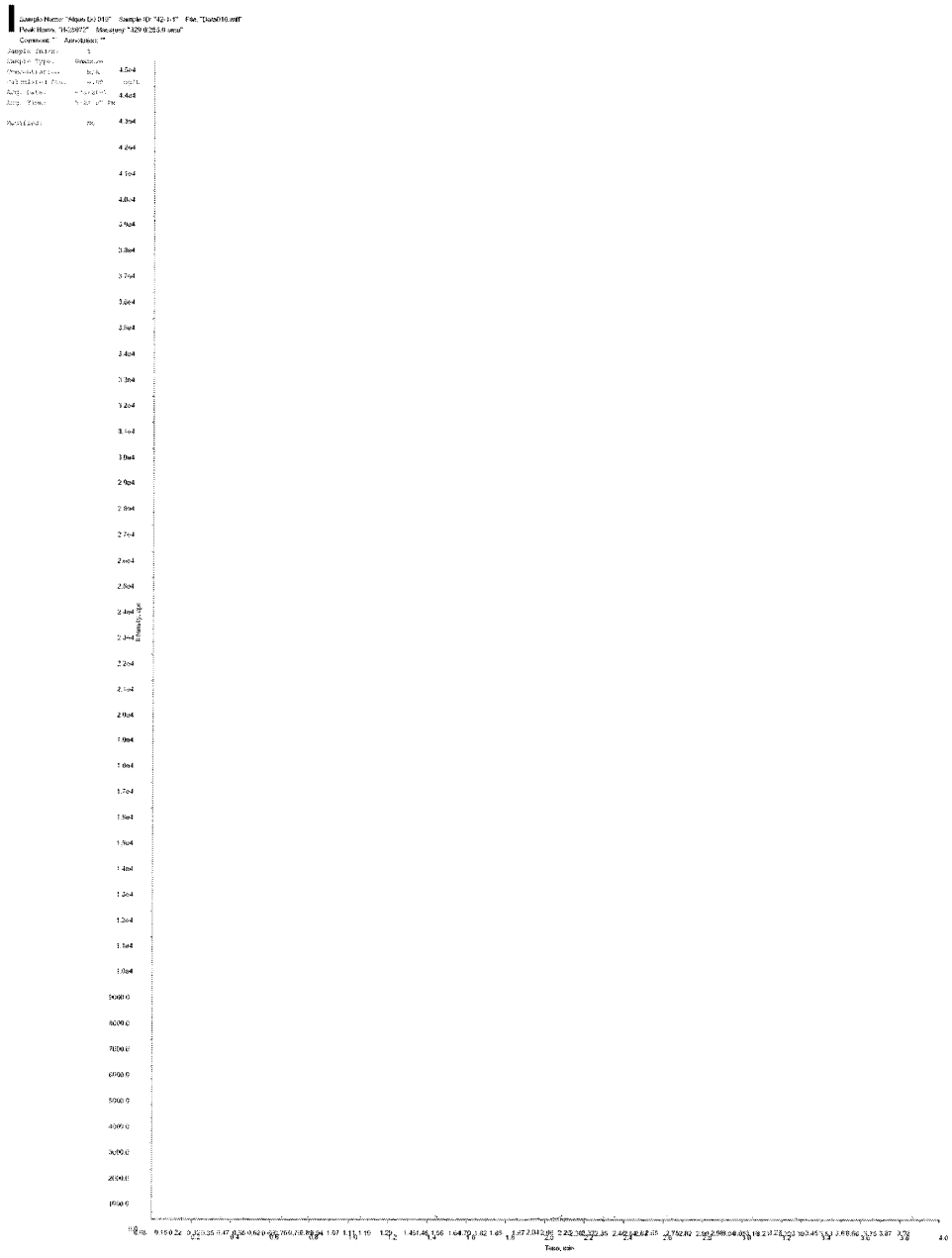
REPRESENTATIVE CHROMATOGRAM OF A CALIBRATION STANDARD SOLUTION



H-28072 elutes at a retention time of approximately 2.09 minutes. The calibration standard solution contained H-28072 at a concentration of 51.8 µg/L.

FIGURE 3

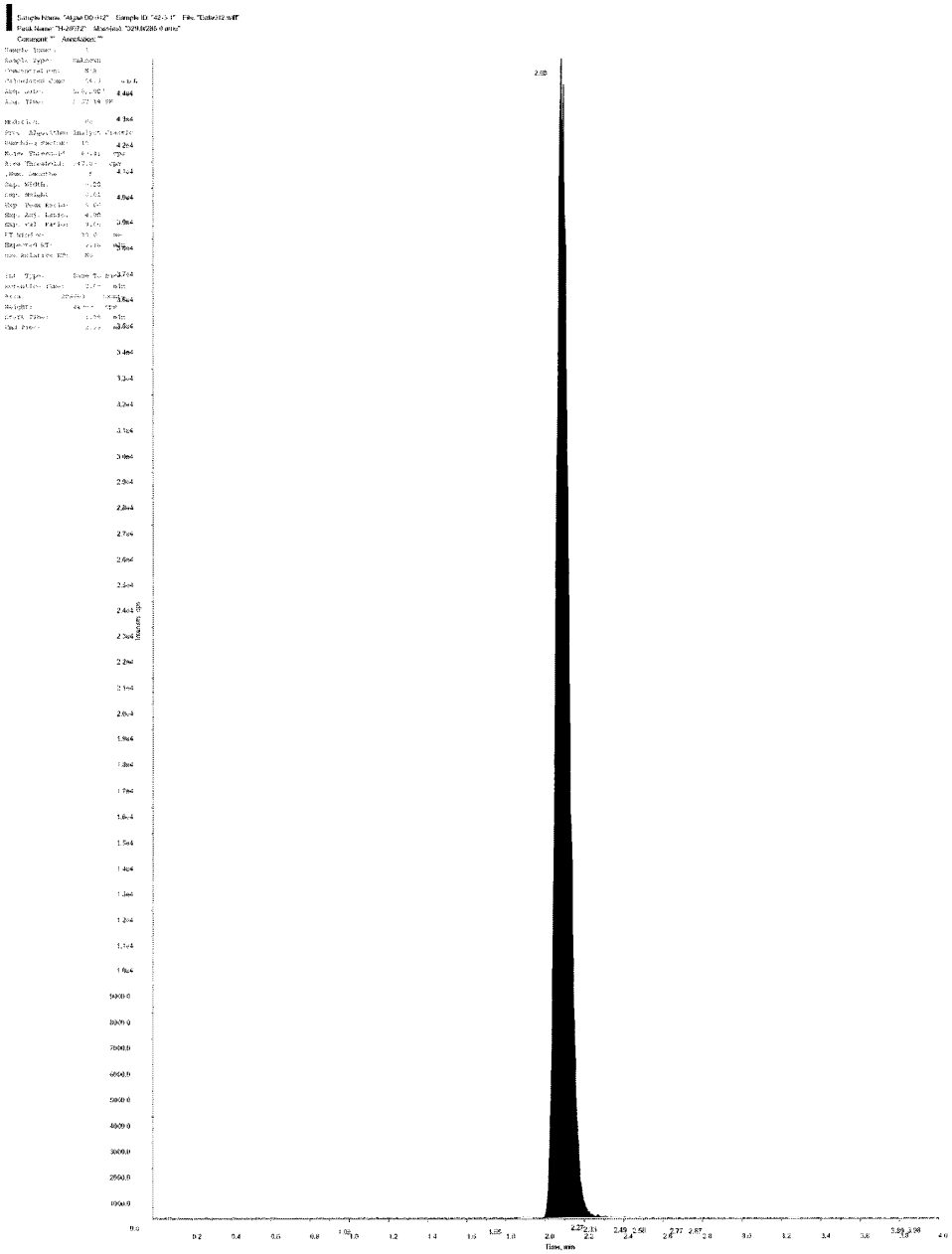
REPRESENTATIVE CHROMATOGRAM OF A BLANK CONTROL SOLUTION



H-28072 would elute at a retention time of approximately 2.09 minutes.

FIGURE 4

REPRESENTATIVE CHROMATOGRAM OF A H-28072 TEST SOLUTION



H-28072 elutes at a retention time of approximately 2.08 minutes. The test solution sample contained H-28072 at a nominal concentration of 60 µg/L. The sample was diluted 2000x with acetonitrile/water, 50/50, v/v, prior to analysis.